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Exposure time relevance of response to nitrite exposure: Insight from transcriptional responses of immune and antioxidant defense in the crayfish, *Procambarus clarkii*

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ABSTRACT

To understand the toxic effects of nitrite exposure on crayfish, expression of genes involved in the immune system, the antioxidant defense, and the heat shock protein 70 (HSP70) was measured after 12, 24, and 48 h of different nitrite concentrations exposure in the hepatopancreas and hemocytes of *Procambarus clarkii*. Nitrite exposure up-regulated mRNA levels of cytoplasmic Mn superoxide dismutase (cMn-SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione-S-transferase (GST), after 24 h nitrite exposure. At 48 h, nitrite exposure decreased the mRNA levels of mitochondrial MnSOD (mMn-SOD), CAT, and GPx. High concentrations of nitrite at 48 h of exposure decreased expression of β-1,3-glucan-bingding protein in the hepatopancreas, and lysozyme expression in hemocytes. Nitrite exposure caused little effect on the heat shock protein 70 (HSP70) in hemocytes. Through overall clustering analysis, we found that 24 h of nitrite exposure caused stronger transcriptional responses. Our study indicated that the response of *P. clarkii* to acute nitrite exposure was exposure time-dependent. These results will help to understand the dynamic response pattern of crustaceans to nitrite pollution, and improve our understanding of the toxicological mechanisms of nitrite in crustaceans.

1. Introduction

An important aspect of aquatic ecotoxicology is to investigate the effects of pollution on aquatic species and to reveal biological response mechanisms. Nitrite is one of the most common pollutants in water systems, which accumulates by bacterial nitrification of ammonia, or by denitrification of nitrate (Martinez and Souza, 2002). Nitrite concentrations are increasing in freshwater ecosystems as a consequence of several anthropogenic sources, such as effluents from industries producing metals, dyes, and celluloids; urban sewage effluents; and aquaculture (Camargo and Alonso, 2006; Jensen, 2003). In some eutrophic shallow lakes, an increased concentration of nitrite is usually observed during the degradation of cyanobacterial blooms (Qin et al., 2007). For example, nitrite nitrogen (nitrite-N) reaches more than 2 mg L^{-1} in eutrophic shallow freshwater lakes (Qin et al., 2007) and 4.61 mg L^{-1} in grow-out white shrimp aquaculture farms (Romano and Zeng, 2013). In wastewater, nitrite concentration can also exceed 1 mg L^{-1} and reach hundreds of mg·L^{-1} nitrite nitrogen, produced by some sections of engineering plants (Kocour Kroupová et al., 2016).

Nitrite is toxic because it is a competitive inhibitor of chloride uptake, which decreases extracellular and intracellular chloride (Lewis and Morris, 1986). For branchial breathers, such as fish or crayfish, nitrite enters into organisms through the eosinophilic chloride-secreting gill cells, which undertake the exchange of Cl^-/HCO_3^- ions (Kocour Kroupová et al., 2016). These animals will take in nitrite at the expense of chlorides and be more sensitive to nitrite exposure with lower ambient chloride concentrations. Furthermore, there is also another route of nitrite uptake by fish or crayfish that diffusion of uncharged nitrous acid across gills (Kocour Kroupová et al., 2016). Nitrite can cause stress response, retardation of growth, damage to various organs, decrease in the tolerance to bacterial or parasitic diseases, and high mortality, by oxidizing hemoglobin, affecting ion level regulation and cardiovascular functions (Camargo and Alonso, 2006; Pottinger, 2017; Romano and Zeng, 2009). An important consequence of nitrite toxicity to aquatic organisms is the overproduction of reactive oxygen species (ROS) and following oxidative damage to macromolecules, including DNA,

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proteins and lipids (Xian et al., 2011). Thus, an effective antioxidant response is essential for aquatic organisms to cope with nitrite exposure. The antioxidant defense system is composed of antioxidant enzymes and low-molecular weight antioxidant scavengers (Hermes-Lima and Zenteno-Savín, 2002). Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) can clear superoxide and hydrogen peroxide. Similarly, glutathione-S-transferase (GST) works in biological detoxication in the presence of glutathione (Nebert and Vasiliou, 2004). Another important adverse effect of nitrite exposure in aquatic organisms is the alteration of the immunity of aquatic organism against aquatic bacteria (Ellis et al., 2011). In Crustaceans, the immune process involves reorganization of pathogen-associated molecular patterns with pattern recognition receptor (PRP) and the activation of phenoloxidase (Akira et al., 2006; Janeway Jr and Medzhitov, 2002; Medzhitov and Janeway, 1997). In addition, the immune system may produce ROS to kill pathogens (Johansson et al., 2000). Therefore, responses of the immune system and the antioxidant defense may be potentially related. Maintaining the protein or transcriptional levels of genes involved in these functions is essential for survival of these species, which may call for heat shock proteins to maintain protein structure or function and to maintain these important functions (Christians et al., 2002).

Several studies have described the response of aquatic organisms to nitrite exposure at the molecular or enzyme activity levels. For example, a previous study generally revealed the molecular response in the hepatopancreas of freshwater prawn *Macrobrachium nipponense* to 24 h or 48 h of nitrite exposure using comparative transcriptome analysis (Xu et al., 2016c; Yu et al., 2019). It was also reported that nitrite exposure increased the antioxidant response or HSP70 expression in hemocytes of white shrimp *Litopenaeus vannamei* or in the gill of red claw crayfish *Cherax quadricarinatus* (Guo et al., 2013a; Jiang et al., 2014). However, for the prevention and cure against nitrite exposure on aquatic organisms by increasing chlorine concentration or moving animals to clean water in time, it is essential to understand the dynamic process of aquatic organism dealing with nitrite exposure with changes in exposure times or pollutant concentrations, to reveal the linkage of different functions, such as the antioxidant defense of immune system, and to analyze the sensibility of aquatic organism to different phases of nitrite exposure. Consequently, there is a need to analysis the integrative transcriptional response patterns involved in important functions in different tissues of aquatic organisms after nitrite exposure, and to address means to their potential role in nitrite toxicity.

The red swamp crayfish *Procambarus clarkii* is a native species of eastern North America that has been introduced worldwide due to its economic and alimentary value. It is considered among the most important commercial species of crayfish (Hobbs et al., 1989). Because crayfish are relatively more sensitive to nitrite toxicity than other aquatic animals (Kocour Kroupová et al., 2016) and because *P. clarkii* is widely distributed in worldwide aquatic system, this species is a good model to study nitrite toxicity. This study aimed at revealing the toxic effects of nitrite exposure along with different nitrite concentrations and exposure times on *P. clarkii* and understanding the dynamic process involving in transcriptional response of *P. clarkii* to nitrite exposure. Expression changes of multiple genes involved in antioxidant defense and immune system in two important tissues (hemocyte and hepatopancreas) of *P. clarkii* after nitrite exposure at different concentrations at different exposure times were measured. The expression of HSP70 in hemocytes was also measured.

2. Materials and methods

2.1. Test organisms

Crayfish juveniles (around 3 months old) were obtained from the Institute of Freshwater Fisheries in Nanjing, China. They were transported to the aquatic laboratory of Nanjing Normal University, where they were held in plastic tanks (140 × 60 × 70 cm) with 20 L aerated tap water at 26 ± 1 °C, pH 7.5 ± 0.5, a dissolved oxygen concentration of approximately 5.0 mg L^{-1} and a 12 h light/dark photoperiod. The crayfish were fed twice a day on commercial pellet diet (Taizhou Chia Tai Co., Ltd) at about 4% of their body weight. Each day, 30% of the water was replaced and all fecal matter was removed.

2.2. Nitrite exposure and collection of hemocytes and the hepatopancreas tissue

After four weeks of acclimation, 90 crayfish (mean weight 25 ± 2 *g*) at intermolt stage were randomly selected for nitrite exposure. Feeding was terminated 24 h before the tests. Based on 96 h LC_{50} value of nitrite exposure (Liu et al., 1995), four levels of nitrite nitrogen (NN, NO_2^--N = 0.5, 1, 2 and 4 mg L^{-1}) plus one control group were used for 48 h toxicity tests with sodium nitrite added. Each treatment was performed in three parallel tanks, with six individuals per tank. The chloride concentrations were setting at 20 mg L^{-1}, which is the common concentration in aerated tap water. The nitrite and chloride concentrations were measured every 8 h and adjusted to the pre-setting value by adding stock solutions of sodium nitrite and sodium chloride. The test solutions were replaced every 24 h using water pump. Dissolved oxygen concentration was approximately 5.0 mg L^{-1}. After 12, 24, and 48 h, three crayfish from each concentration group were randomly removed and anesthetized on ice. Hemolymph samples were taken individually from the base of walking legs with a 1 mL sterile syringe. Half of the hemolymph was diluted with an equal volume of anticoagulant solution (4.8 *g*·L^{-1} citric acid, 13.2 *g*·L^{-1} sodium citrate, 14.7 *g*·L^{-1} glucose), centrifuged at 1000 × g at 4 °C for 5 min to collect the cells, and then washed twice in phosphate-buffered saline (Xia et al., 2017; Zheng et al., 2017). The hepatopancreas was removed for RNA isolation.

2.3. RNA isolation

Total RNA of hemocytes and hepatopancreas was extracted using TRIzol reagent (TaKaRa, Japan), according to the manufacturer's protocol. Briefly, the samples were cracked, and then RNA was extracted by chloroform, precipitated by isopropanol, and washed by 75% ethyl alcohol. RNA quality was assessed using electrophoresis on a 1.2% agarose gel. Total RNA concentration was determined by measuring the absorbance at 260 nm on a spectrophotometer. RNA with good integrity and 260/280 ratio between 1.8 and 20 was used for cDNA synthesis.

2.4. cDNA synthesis and gene expression assay

A total of 5 μg of RNA was reverse transcribed using the PrimerScript™ First Strand cDNA Synthesis kit (TaKaRa, Japan), according to the manufacturer's instructions.

Expression of 10 target genes: mitochondrial Mn superoxide dismutase (mMn-SOD), cytoplasmic MnSOD (cMn-SOD), extracellular Cu/Zn-SOD (exCu/Zn-SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), phenoloxidase (PO), lysozyme, β-1,3-glucan-bingding protein (LGBP), heat shock protein 70 (HSP70), and the internal control gene, β-actin, was measured by quantitative real-time PCR. Gene expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The specific primers used in the present study are shown in Table 1.

2.5. Statistical analysis

When normality and homogeneity of variances were appreciated, the data on gene expression were evaluated by two-way analysis of variance (ANOVA), followed by Tukey HSD tests ($\alpha = 0.05$). When the requirements of normality and homogeneity of variances were not met, the non-parametric Kruskal-Wallis H-test was used, followed by Mann-Whitney *U*-test. To overview global change patterns, the Euclidean

Table 1
Primers used in the present study.

Gene	Sequences (5'-3')	GenBank number
β-actin	F: TGCGACTCTGGTGATGGTGT R: AGCGGTGGTGGTGAAGGAAT	KR135165.1
cytoplasmic MnSOD	F: CTGCAGCCAGTGTTGGAGTGAA R: AAGGGAATCAGACCGTGAGTGATC	EU254488.3
mitochondrial MnSOD	F: AGTTTCAGCCGTCTGTTCGC R: ACGGGTTCTAAAGCATTGTAGTCA TGGTGGTGTTTGGAGTGATGCAGTTG	KC333178.1
extracellular Cu/Zn-SOD	F: CAAATCAGTGGCAGGCTGGAAA R: TCGCCCAGGTCACCCTTCTCG	KC333177.1
CAT	F: GCTGAGGTGGAACAGATGGCA R: AAGGGAATCAGACCGTGAGTGATC	KM068092.1
GPx	F: CCGCTCTTCACCTTCTTG R: GCGAGTGTATGGCTTACC	JN835259.1
GST D1	F: ACCTGCCATATTACATTGAC R: CCTTATCTTCTCTTGCTCTG	HQ414581.1
PO	F: AGTTCGGGCTGGATG A R: AATGCGTCTGCTAAGATGTC	EF595973.1
Lysozyme	F: TACTTATTGGGTTGACGGCTGCTA R: AGTGGACGCATCGCACAAGC	GQ301200.1
LGBP	F: GGCAGACTAACTTGTATGAGAG R: GTCCACGAACACCTGTATG	FJ410910.1
HSP70	F: ACCTGCCATATTACATTGAC R: CCTTATCTTCTCTTGCTCTG	DQ301506.1

method was used in hierarchical clustering algorithm with all standardized data. Gene expression was standardized to the value of fold change, and then mapped to the interval (-1/1) proportionally with maximum net fold change as 1, in which down-regulated genes are represented by negative values and the up-regulated genes by positive values. All data are presented as mean ± S.E. One-way ANOVA was used to compare inter-group differences according to the results of clustering analysis. All statistical analyses were carried out with SPSS (ver. 19, SPSS Inc., USA); the clustering analysis was performed with R v. 3.5.0 (Development Core Team 2018).

3. Results

3.1. Effect of nitrite exposure on gene expression changes

Two-way ANOVA showed that there was an interaction between exposure time and nitrite concentration on expression of exCu/ZnSOD in the hepatopancreas, which was mainly affected by nitrite concentration (Fig. 1A). At 4 mg L^{-1} NN, increased exCu/Zn-SOD mRNA levels were observed after 12 h and 24 h of exposure, compared to respective control group (one-way ANOVA: $P_{12h} = 0.001$ and $P_{24h} < 0.001$). After 48 h of exposure, both 1 and 2 mg L^{-1} NN nitrite increased exCu/Zn-SOD mRNA levels; this increase was not observed at 4 mg L^{-1} NN (one-way ANOVA: $P < 0.001$). Nitrite concentration affected expression of mMn-SOD, which was also induced by exposure time, and interaction between nitrite concentration and exposure time was observed (Fig. 1B). The mMn-SOD mRNA did not change at all concentrations after 12 h of exposure (one-way ANOVA: $P = 0.071$). After 24 h and 48 h of nitrite exposure, moderate nitrite concentrations (0.5 mg L^{-1} NN after 24 h and 1 mg L^{-1} NN after 48 h) caused up-regulation of mMn-SOD mRNA (one-way ANOVA: $P_{24h} < 0.001$ and $P_{48h} < 0.001$). Both nitrite concentration and exposure time affected the expression of cMn-SOD and also showed an interaction (Fig. 1C). At 0.5 mg L^{-1} NN, nitrite exposure decreased cMn-SOD mRNA after 12 h of exposure (one-way ANOVA: $P = 0.002$). Nitrite of 0.5 and 1 mg L^{-1} increased cMn-SOD mRNA levels after 24 h of exposure; then this change recovered (one-way ANOVA: $P = 0.008$). After 48 h of nitrite exposure, 2 and 4 mg L^{-1} NN nitrite exposure decreased cMn-SOD mRNA levels (one-way ANOVA: $P = 0.030$).

Expression of CAT in the hepatopancreas was affected by nitrite concentration and showed the higher expression level in 24 h group (Fig. 1D). Nitrite concentration and exposure time showed an interaction (Fig. 1D). Compared with the control group, only 0.5 mg L^{-1} NN nitrite exposure increased CAT mRNA level after 12 h and 48 h while all concentrations of nitrite up-regulated CAT expression after 24 h of exposure (one-way ANOVA: $P_{12h} < 0.001$, $P_{24h} < 0.001$, and $P_{48h} < 0.001$). Two-way ANOVA showed a similar result of expression of GPx and GST with that of CAT (Fig. 1E and F). After 12 h of nitrite exposure, GPx mRNA increased at 4 mg L^{-1} and up-regulated at all nitrite concentration treatments after 24 h of exposure (one-way ANOVA: $P_{12h} < 0.001$ and $P_{24h} = 0.004$). After 48 h of exposure, only nitrite exposure of 1 mg L^{-1} increased GPx expression (one-way ANOVA: $P < 0.001$). Both 0.5 and 1 mg L^{-1} NN nitrite caused elevation of GST mRNA after 12 h of exposure and all concentrations of nitrite induced GST mRNA expression after 24 h of exposure (one-way ANOVA: $P_{12h} < 0.001$ and $P_{24h} = 0.001$). GST mRNA did not change in all concentrations of nitrite after 48 h of nitrite exposure (one-way ANOVA: $P = 0.532$).

Two-way ANOVA indicated that the both nitrite concentration and exposure time affected expression of PO and also showed an interaction (Fig. 1G). After 12 h and 24 h of nitrite exposure, only 4 mg L^{-1} NN nitrite caused elevation of PO mRNA level in the hepatopancreas (one-way ANOVA: $P_{12h} = 0.001$ and $P_{24h} < 0.001$); while this mRNA level did not change after 48 h of nitrite exposure (one-way ANOVA: $P =$

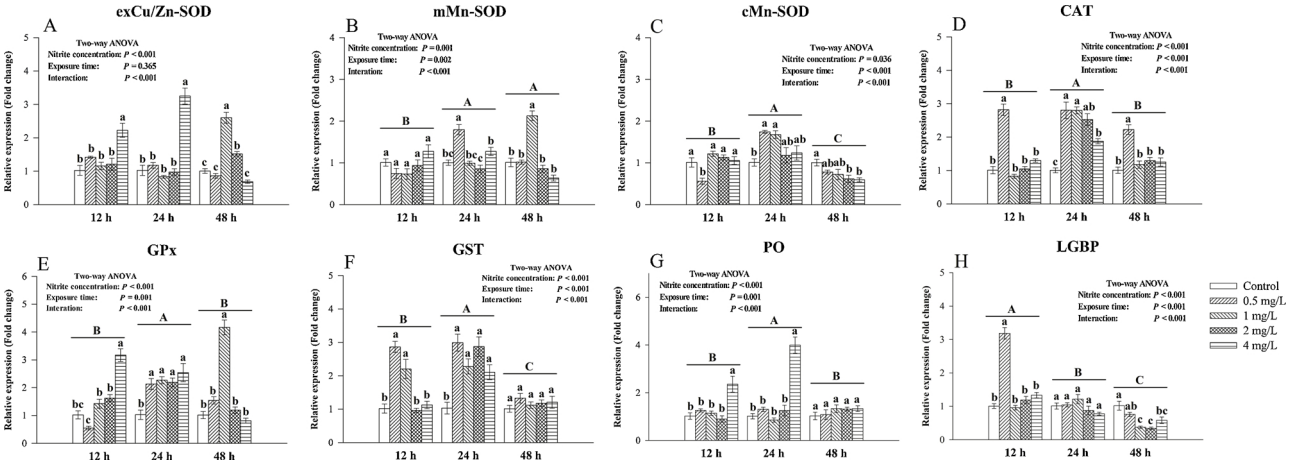

Fig. 1. Effect of acute nitrite exposure on expression of genes involved in immune system and the antioxidant defense in the hepatopancreas of *Procambarus clarkii*. Data were represented as mean ± s.e. Within each set of exposure time groups, data with no common superscript lowercase letters indicate a significant difference between different nitrite concentration groups ($P < 0.05$). The different capital letters indicate the significant difference between different exposure time ($P < 0.05$).

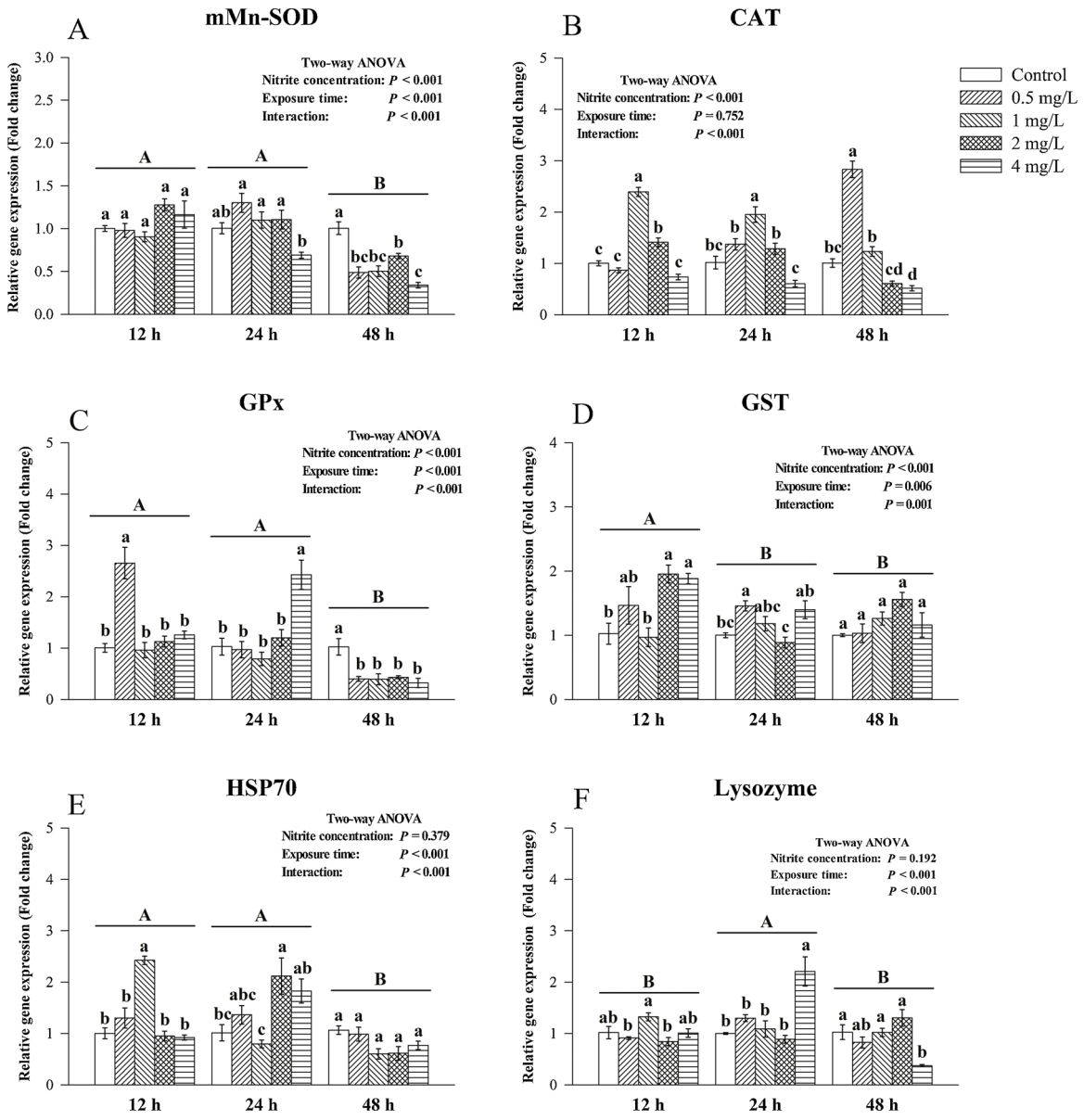

Fig. 2. Effect of acute nitrite exposure on expression of genes involved in the immune system, the antioxidant defense, and HSP70 in hemocytes of *Procambarus clarkii*. Data are represented as mean ± s.e. Within each set of exposure time groups, data with no common superscript letters indicate a significant difference between nitrite concentration groups ($P < 0.05$). The different capital letters indicate the significant difference between different exposure time ($P < 0.05$).

0.436). Exposure time depressed the expression of LGBP (Fig. 1H). After 12 h of nitrite exposure at 0.5 mg L^{-1} NN, LGBP mRNA increased ; and LGBP mRNA level did not change after 24 h of nitrite exposure (one-way ANOVA: $P_{12h} < 0.001$ and $P_{24h} = 0.083$). After 48 h of nitrite exposure, LGBP mRNA level was reduced in 1, 2, and 4 mg L^{-1} (one-way ANOVA: $P < 0.001$).

Long-term (48 h) exposure inhibited the expression of mMnSOD in the hemocyte and interact with nitrite concentration (Fig. 2A). Relative to control group, mMn-SOD mRNA did not change at all nitrite concentrations after 12 h and 24 h of exposurewhile decreased at all nitrite concentrations after 48 h of exposure (one-way ANOVA: $P_{12h} = 0.085$, $P_{24h} = 0.008$, and $P_{48h} < 0.001$). Nitrite concentration significantly affected expression of CAT and interact with exposure time, which did not affect CAT mRNA solely (Fig. 2B). CAT mRNA increased at 1 and 2 mg L^{-1} NN nitrite concentrations after 12 h of exposure (one-way ANOVA: $P < 0.001$), and this increase was also observed at 1 mg·L^{-1} NN nitrite concentration after 24 h of exposure in hemocytes (one-way ANOVA: $P < 0.001$). After 48 h of exposure, 0.5 mg·L^{-1} NN nitrite up-regulated CAT mRNA while 4 mg L^{-1} NN nitrite decreased the expression (one-way ANOVA: $P < 0.001$). Expression of GPx and GST in the hemocyte was affected by nitrite concentration, inhibited by exposure time, and affected by interaction of both two factors (Fig. 2C and D). At 12 h of nitrite exposure, low concentration nitrite exposure (0.5 mg L^{-1} NN), and high concentration nitrite at 24 h of exposure (4 mg L^{-1}) up-regulated GPx mRNA in hemocytes (one-way ANOVA: $P_{12h} < 0.001$ and $P_{24h} = 0.001$). After 48 h of nitrite exposure groups, GPx expression decreased in all concentration groups (one-way ANOVA: $P = 0.003$). Nitrite exposure of 2 and 4 mg·L^{-1} caused up-regulation of GST mRNA after 12 h of exposure, and this up-regulation was also observed after 24 h of exposure to 0.5 mg L^{-1} NN nitrite (one-way ANOVA: $P_{12h} = 0.007$ and $P_{24h} = 0.007$). Expression of HSP70 and lysozyme in the hemocyte were mainly affected by exposure time, which interacted with nitrite concentration (Fig. 2E and F). HSP70 mRNA was up-regulated after 12 h of exposure to 1 mg L^{-1} NN nitrite and after 24 h of exposure to 2 mg L^{-1} NN nitrite; while expression of this gene did not change at 48 h of exposure to all nitrite groups (one-way ANOVA: P_{12h}

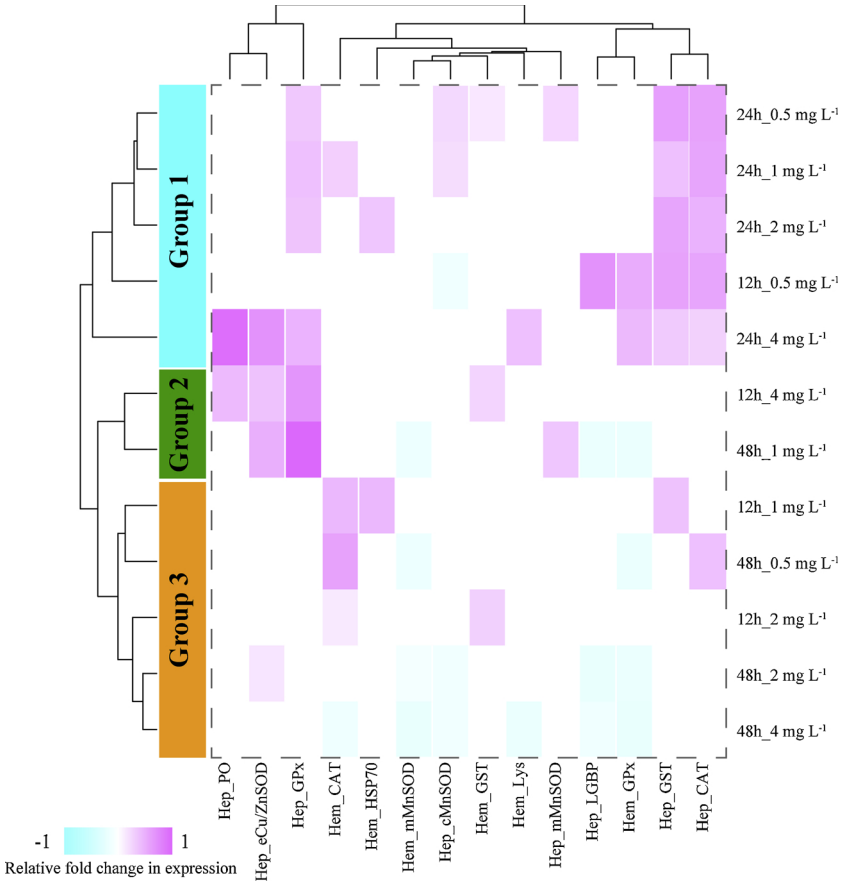

Fig. 3. Clustering results of the overall response pattern of standardized gene expression in *Procambarus clarkii* after nitrite exposure. Treatments were clustered into three groups (groups 1–3). The pink block indicated up-regulated genes and the blue block indicated down-regulated genes. The white block indicated the genes did not show significantly different expression changes.

< 0.001, $P_{24h} = 0.009$, and $P_{48h} = 0.040$). Only 4 mg L^{-1} NN nitrite increased lysozyme mRNA after 24 h of exposure ; after 48 h of exposure, 4 mg L^{-1} NN nitrite strongly decreased lysozyme mRNA level (one-way ANOVA: $P_{24h} = 0.001$ and $P_{48h} = 0.002$).

3.2. Effect of nitrite exposure on overall expression change pattern

Clustering result was in Fig. 3. According to the relative expression change of each gene at different nitrite concentrations and at different exposure times, the treatments were clustered into three groups (Fig. 3). Group 1 included all nitrite concentrations at 24 h of exposure and the 12 h 0.5 mg mg·L^{-1} exposure group; the data in this group showed higher relative expression changes than that data in group 2 or 3, which were at 12 h or 48 h of exposure to nitrite ($P = 0.003$).

4. Discussion

Nitrite exposure in aquatic ecosystems can induce the formation of ROS and RNS in organisms, which then affects the antioxidant system and other macromolecules (Jensen et al., 2015; Jensen and Hansen, 2011; Sun et al., 2014). In addition, Nitrite exposure oxidizes hemoglobin in hemocytes, increases the amount of methemoglobin, and then causes hypoxia in organisms, which may cause overproduction of ROS (Cheng et al., 2013; Meinelt et al., 2010). As the first line in antioxidant defense, SOD plays important role in response to O_2^- by catalyzing superoxide anion radical into molecular oxygen and hydrogen peroxide (Sies, 1993). Different types of SOD were identified in crustaceans with different metallic ions at the active site (Plantivaux et al., 2004). In the present study, short-term nitrite exposure (12 h) caused little effect on SOD mRNA level, while medium-term nitrite exposure (24 h) stimulated SOD expression, especially of cMn-SOD, in the hepatopancreas. This indicated formation of excess cytoplasmic O_2- in the hepatopancreas of *P. clarkii* after 24 h of nitrite exposure. In the gill of crayfish *Cherax quadricarinatus*, nitrite exposure induced SOD mRNA after 12 h and 24 h (Jiang et al., 2014). This may indicate that the transcriptional response of SOD may be related with the way tissues contact nitrite, as the gill can contact nitrite more directly and there may be a time lag for the hepatopancreas. Our results also indicated that long-term nitrite exposure may depress expression of SOD isoforms in tissues of *P. clarkii*, as that, after 48 h of exposure, high concentrations of nitrite decreased cMn-SOD mRNA in the hepatopancreas, and mMn-SOD mRNA level in hemocytes. It has been reported that SOD activity in the shrimp *Macrobrachium nipponense* decreased with increasing nitrite concentrations at 24 h of exposure (Wang et al., 2004), which was also reported in hemocytes of *Macrobrachium rosenbergii* (Zhang et al., 2015). However, these results did not completely indicate the effect of exposure time on SOD activities. In our study, reduced SOD expression appeared with the prolonged exposure time, but not from the start of the exposure in *P. clarkii*.

Both CAT and GPx can degrade hydrogen peroxide to remit oxidative stress. In the present study, the response of CAT and GPx to oxidative stress occurred in the hepatopancreas after 24 h of nitrite exposure. These results, combined with the results of cMn-SOD, may indicate that the peak of oxidative stress occurred after 24 h of nitrite exposure. In the gill of shrimp *C. quadricarinatus*, GPx mRNA responded to nitrite exposure after 12 h of exposure (Jiang et al., 2014). Similar with the change in SOD expression, this may also indicate a possible tissue-specific response time lag, which makes CAT and GPx in the hepatopancreas respond to nitrite exposure at a later stage. As exposure time increased, nitrite at low concentrations induced higher CAT or GPx mRNA levels while, at high concentrations, we found that CAT or GPx mRNA did not change. This may be attributed to that long-term exposure at high nitrite concentration promoted a more rapid adaptation in *P. clarkii*, while long-term accumulation effect of low concentration

of nitrite increased CAT or GPx expression. However, in hemocytes, a suppression on the antioxidant defense in hemocytes of *P. clarkii* after 48 h of exposure was observed, indicating a tissue-specific effect.

The expression of GST was up-regulated after 12 h of exposure to low nitrite concentrations and, then showed a strong response to 24 h of nitrite exposure in the hepatopancreas, indicating a need for detoxication to mitigate the toxicity of nitrite exposure. Compared with the expression changes of other antioxidant genes, nitrite exposure caused little effect on GST expression in hemocytes. RNA-seq studies showed the need for detoxification response, including induced GST expression, in *Litopenaeus vannamei* responding to nitrite exposure (Guo et al., 2016; Guo et al., 2013b). Our results indicated that this need for GST in *P. clarkii* changes with exposure time, especially after 24 h of exposure.

Immune response is an important response to nitrite exposure in invertebrates (Guo et al., 2016, 2013b). β-1,3-glucan-bingding protein (LGBP) is one of PRPs and can activate the proPO system to PO (Akira et al., 2006; Lee et al., 2003; Söderhäll and Cerenius, 1992). Nitrite at high concentrations stimulated PO expression in the hepatopancreas of *P. clarkii* after 12 and 24 h of exposure. However, PO expression recovered after 48 h of nitrite exposure, which may be attributed to depressed LGBP mRNA in the hepatopancreas. This may result in decreased immunity of *P. clarkii* after long-term nitrite exposure despite constant PO expression level. Decreased PO activity was reported in *L.vannamei* and *M. malcolmsonii* after 48 h of nitrite exposure and these organisms showed sensibility to bacteria accordingly (Chand and Sahoo, 2006; Tseng and Chen, 2004). Our results suggested that decreased LGBP mRNA after 48 h of nitrite exposure may contribute to depressed immunity in crustaceans. We found that 24 h of exposure to 4 mg L^{-1} NN nitrite stimulated lysozyme expression in hemocytes while after 48 h its expression was decreased. Lysozyme, widely distributed in eukaryotes and prokaryotes, hydrolyses bacterial cell walls and plays an important role in innate immunity (Zhao et al., 2012). The change in lysozyme expression suggested that a high level of nitrite had a negative effect on tolerance to bacterial diseases. As reported in other studies that both acute and chronic nitrite exposure affected immune system in *Macrobrachium nipponense*, longer-term nitrite exposure decreased the immune function in *P. clarkii* (Xu et al., 2016c). Environmental stress up-regulated HSP70 expression to maintain homeostasis by preventing protein denaturation (Christians et al., 2002; Morimoto and Santoro, 1998). In the present study, nitrite exposure at 12 h 1 mg L^{-1} NN and 24 h 2 mg L^{-1} NN increased HSP70 mRNA levels in hemocytes of *P. clarkii* and, importantly, no decreased HSP70 expression was observed. Several studies showed that HSP70 levels increased in fish during nitrite exposure to prevent tissue damage (Deane and Woo, 2007; Jensen et al., 2015). In a marine crab, HSP70 expression increased strongly during short-term nitrite exposure, but not during long-term nitrite exposure (Xu et al., 2016a). Thus, enhanced or constant HSP70 expression is essential for aquatic organisms to respond to nitrite exposure. Interestingly, the main changes in HSP70 expression were observed after 12 h or 24 h of exposure, but not after 48 h of exposure. This may indicate a possible adaptation of HSP70 expression in *P. clarkii* to nitrite exposure after a longer time, with that maintaining the stable expression is the main strategy.

In the present study, the transcriptional responses at different nitrite exposure times were highly variable, especially a stronger response after 24 h of exposure to nitrite (Fig. 3). This suggested that the immune or antioxidant response pattern of *P. clarkii* to nitrite exposure may be related to exposure time rather than to the nitrite concentration. This may indicate that the accumulative effect of nitrite exposure over time is stronger than the simple short-term concentration effect. The mechanisms of nitrite toxicity involved in competing with chloride uptake in the gill and then, nitrite accumulation or chloride loss (Xu et al., 2016b). At the early phase during nitrite exposure, the effect of nitrite may work on peripheral tissues, such as gills (Jiang et al., 2014). With the accumulation of nitrite, hypoxia may occur, and induce oxidative stress, and then corresponding antioxidant response (Fig. 3). Notably, enhanced immune gene expressions can also be observed here rather than being decreased throughout. After longer-term exposure to nitrite, decreased gene expression occurred, even at the lowest nitrite concentration (Fig. 3). This nitrite exposure time relevance of response in *P. clarkii* matched time and intensity-based classifications of oxidative stress in environmental research (Lushchak, 2016). Therefore, in crayfish culture, to prevent against the accumulative effect of nitrite, other protective ways, such as antioxidant supplement, are still needed in addition to maintaining low concentration of nitrite.

5. Conclusion

Our results clearly revealed stronger response of most genes involved in the antioxidant defense, the immune system, and HSP70 to 24 h of 0.5, 1, 2, or 4 mg L^{-1} NN concentrations exposure in the hepatopancreas and hemocytes of *P. clarkii*. This response will cease after 48 h of nitrite exposure. Our study indicated a nitrite exposure time relevance of integrative response pattern. Nitrite exposure caused little effect on HSP70 mRNA levels, which was an essential strategy to cope with nitrite exposure. Our results provided further evidence to reveal that how nitrite exposure affect antioxidant defense and immune function in crustaceans, which may contribute to nitrite toxicity.

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